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Isolation, molecular identification and screening of halophilic and thermophilic-lipase producing bacterial strains from extreme environmental conditions

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ABSTRACT

Aims: The aim of this study was to isolate and screen lipolytic-thermophilic and halophilic strains from extreme environments and at the same time studying the influence of physiological conditions such as temperature and sodium chloride concentrations on growth of the strains.
Methodology and results: A total of 45 halophilic bacterial strains and 4 thermophilic bacterial strains were isolated from various saline environments and hot spring respectively, and were screened for lipolytic activity on tributyrin-containing agar. The strains that showed lipolytic potential on the solid agar were further subjected to secondary screening by submerged fermentation using the same substrate (tributyrin). One halophilic (SWJ2) and thermophilic (HS2) strain each with highest extracellular lipase production ability was outsourced for molecular identification. The result from 16S rRNA partial gene sequencing and amplification of the selected isolates designated the halophilic strain SWJ2 as *Marinobacter* sp. SWJ2 and the thermophilic strain HS2 as *Bacillus* sp. HS2. Upon screening under submerged fermentation, *Bacillus* sp. HS2 gave 0.17 U/mL lipase production while *Marinobacter* sp. SWJ2 had 0.57 U/mL lipase production.
Conclusion, significance and impact of study: It could be inferred that the selected strains possess high lipase producing potential with interesting thermal and halophilic properties. Continuous screening of extremophilic environment for strains producing lipases with novel properties will improve their potential industrial and biotechnological applications especially for those processes conducted in harsh reaction conditions.

Keywords: Bacillus sp.HS2, *Marinobacter* sp.SWJ2, extremophilic

INTRODUCTION

Recently, several industrial applications demand that industrial enzymes must be stable at extremes of temperature, PH, and salt concentration. Therefore, isolation of lipase from extremophiles may yield useful enzyme for the aforementioned purpose. Extremophiles are organisms that have evolved to exist in a variety of extreme environments and they fall in a number of different classes that include thermophiles, halophiles, acidophiles, alkaliphiles, psychrophiles, and barophiles (Fuciños *et al.*, 2012). They have the potential to produce uniquely valuable biocatalysts that function under conditions in which the enzymes of their non-extremophilic counterparts could not. This research article focuses on two of these classes, namely; thermophiles and halophiles.

Thermophiles are microorganisms that are adapted to life at high temperatures in which the optimal growth temperature range is between 45-80 °C and above (López-López *et al.*, 2015). They live in hot springs, submarine hydrothermal vents, geothermal heated oil, petroleum reservoirs, sun-heated soils, terrestrial geothermal and volcanic areas and have been found to produce thermostable lipase (Salihu and Alam, 2014). The inherent thermostability of proteins from thermophiles is attributed to the increased number of stabilizing molecular interactions such as extended ionic networks of hydrophobic interactions, hydrogen bonding, disulphide bonds, shorter surface loops, capped N- and C- terminal as well as rigid and packed conformational structure of the enzymes (Sharma *et al.*, 2013).

Halophilic bacteria are organisms requiring high NaCl for growth or activity while halotolerant bacteria require normal NaCl concentration (0.5-1%) but can also tolerate

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up to 5% (w/v) NaCl concentration. Bacterial halophiles for example, *Salinibacter*, *Bacillus*, *Halobacillus*, *Marinobacter* sp., *Marinococcus*, *Micrococcus*, *Alcaligenes*, and *Pseudomonas* sp. live in environments with high salt concentration that would kill most other microbes. They are abundant in environment such as fresh water, Dead Sea, salt crystallizer ponds, salt lakes, saline soils, and salted food products (Khunt, 2012). Halophiles have been distinguished on the basis of their salt requirement for growth as; slight halophiles, 0.2-0.5 M (1-3%), moderate halophiles, 0.5-2.5 M (3-15%), and extreme halophiles, 2.5-5.2 M (15-32%) (Arora *et al.*, 2014). Two fundamentally different strategies enable halophilic organisms to cope with osmotic stress. They either accumulate internal organic compatible solutes to balance the osmotic stress of the environment or produce acidic proteins to increase solvation and improve high salinity function (DasSarma and DasSarma, 2012). Therefore, enzymes from halophilic organisms are uniquely adapted to function in conditions with low water potential, thus, it is predicted that many of their enzymes are functional in organic and/or hydrophobic solvents (Calimlioglu and Arga, 2014). In addition to the halophilic characteristics of enzymes from halophilic organisms, lipases produced by these organisms have advantages particularly with regards to their thermal stability (Munawar and Engel, 2013; Salihu and Alam, 2015).

Halophilic lipases display polyextremophilicity, that is, stability and activity in more than one extreme condition, such as, high salt concentration, high temperature, alkaline pH, and non-aqueous medium (Karan *et al.*, 2012). These properties give halophilic lipases variety of biotechnological applications, especially catalysing reactions under aggressive conditions such as wide range of pH, high temperature, and where physiochemical conditions are not appropriate for the enzymatic activity of the organism (Delgado-García *et al.*, 2012). Lipases produced by these organisms are utilized in a number of industrial applications, such as food industry for flavour formation and where unique characteristics are required such as biodiesel production and synthesis of fine chemicals.

Lipases catalyze the hydrolysis of triacylglycerol at the oil-water interface to release glycerol and free fatty acid (Dheeman *et al.*, 2010). They are produced by many microbes and higher eukaryotes but the useful ones are of microbial origin. Microbial lipases are produced mostly by submerged fermentation (SMF) and solid state fermentation methods. Lipase activity is determined using agar plates containing substrates for lipases with or without an indicator. The most widely used methods are tributyrin agar plate, calcium-triolein agar plate and Rhodamine B agar plate (Patil *et al.*, 2011).

With the enormous increase in the use of lipase biocatalysts in the industry, especially those processes requiring harsh conditions of elevated temperature, presence of organic solvent and change in pH of the reaction medium, there is need for continuous search for additional novel thermophilic and halophilic strains of highly active lipase with specific stability to high temperature, pH, and ionic strength. This research is focussed on isolation and screening of lipolytic thermophilic and halo-thermophilic strains as well as studying the influence of physiological conditions such as halotolerance and thermotolerance on growth of the strains.

MATERIALS AND METHODS

Collection of samples

Hot water sample was collected in a sterile container from FELDA residence hot spring (Latitude 4.0001° N, Longitude 101.3101° E), Sungkai, Perak, Malaysia. Salty water sample was collected about 50 m from the seacoast of Tanjung Piai National park (Latitude 1.2681° N, Longitude 103.5087° E), Johor Bahru, Malaysia. Salty wastewater (effluent), soil and sludge samples were collected from a local salted fish factory in Kuala Perlis (Latitude 6.3979° N, Longitude 100.1307° E), Perlis, Malaysia. Four different salted fish samples were also collected from local fish market in Kuala Perlis, Perlis, Malaysia and stored at 4 °C in a cold room.

Isolation of halophilic and thermophilic bacterial strains

Halophiles were isolated from sea water, salty wastewater, salted fish, soil and sludge samples. Different dilutions of sea water, salty wastewater and sludge samples were added to the halophilic isolation medium (Modified Sehgal and Gibbons complex medium) and incubated at 37 °C for 96 h. The modified Sehgal and Gibbons Complex (SGC) medium contained (g/L): Casein-peptone: 7.5; Yeast extract: 10.0; KCl: 2.0; Sodium citrate: 3.0; $MgSO_4 \cdot 7H_2O$: 20.0; $FeSO_4 \cdot 7H_2O$: 0.01; Agar: 20.0 supplemented with 3%, 7%, 10%, 15%, 20% and 25% (w/v) NaCl. The medium was adjusted to pH 7.0 by 0.5M NaOH (Anisha *et al.*, 2012). Small pieces of salted fish samples of about 1 g was inoculated into 50 mL of SGC medium contained in 250 mL Erlenmeyer flask. The flasks were incubated on a rotary shaker with a speed of 200 rpm at 37 °C for 7 days until turbidity appeared. The turbid cultures were then streaked on SGC medium containing 2.0% agar and incubated for 7 days (Anisha *et al.*, 2012). Pure colonies were selected and maintained on SGC medium slant, and stored at 4 °C until further use.

Thermophiles were isolated from the hot water sample by serial dilution of the sample up to 10^{-4} and dispensed on nutrient agar plate using spread plate technique. The inoculated plates were incubated at 50 °C for 48 h. Pure plate cultures were prepared and the distinct colonies were maintained on nutrient agar slant, and stored until further use.

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Morphological and physiological characterization of bacterial strains

Morphological and physiological characterizations of the isolated halophilic and thermophilic bacterial strains were performed according to standard method (Kanlayakrit and Boonpan, 2007). The pure cultures of the isolated organisms were characterized in terms of colony morphology on halophilic agar and nutrient agar, Gram's staining, and cell shape as recommended by Olutiola *et al.* (2000). Physiological characterization such as optimum growth temperature and tolerance to NaCl% (w/v) were also routinely determined for the halophilic strains.

Screening of the bacterial strains for lipase production on solid agar

Distinct colonies of halophilic bacterial strains selected on the basis of colony characteristics were screened for lipase production on SGC agar medium containing 1% tributyrin. The halophilic bacterial isolates were inoculated on the agar plates by making a straight streak and were incubated at 37 °C for 72 h. A positive reaction for lipase activity was indicated by the formation of transparent or opaque zone around the colony. The diameter of the colony and zone of clearance around the colony were then measured. Relative enzyme activity (REA) was calculated using the formula (Anisha *et al.*, 2012):

REA = Diameter of clearance zone / Diameter of colony

The isolates showing maximum REA were selected for further studies.

Screening of lipolytic thermophilic bacteria was carried out using tributyrin agar plate method (Bisht and Panda, 2011). Each pure culture was streaked on tributyrin agar plate and incubated at 48 °C for 2 days. Lipase production was indicated by the presence of surrounding clear hydrolytic zones.

Screening of the halophilic bacterial strains for lipase production using submerged fermentation (SMF) technique

Inoculum for halophilic bacteria was prepared by transferring a loop of pure bacteria culture into 25 mL of modified SGC medium without agar (as described earlier) at pH 7.0 in a 100 mL Erlenmeyer flask. The flask was then incubated on a rotary shaker at 37 °C for 48 h. Two millilitre (1.0×10^{-6} cells/mL) of the seed culture was later inoculated into 50 mL modified SGC medium (pH 7.0) supplemented with 2% tributyrin and 10% NaCl for moderate halophiles and 15-20% for extreme halophiles, contained in 250 mL Erlenmeyer flask. The halophilic bacterial culture was incubated at 37 °C for 60 h under static condition and on incubator shaker maintained at 150 rpm. Three millilitre of the culture suspension was taken out after the incubation period and absorbance was measured at 540 nm wavelength using spectrophotometer (UV-VIS Shimadzu, Japan). The culture suspension was then centrifuged at 8,000 rpm for 20 min at 4 °C (Kanlayakrit and Boonpan, 2007) and the supernatant was used as the crude extracellular lipase for lipase activity determination. All experiments were conducted in triplicates.

Table 1: Colony characteristics of the halophilic bacteria on modified Sehgal and Gibbons complex agar.

Strain code	Colony morphology
BSO1	Light-brown, irregular, convex, mucoid, opaque
BSO2a	Light-brown, circular, convex, mucoid, entire
BSL1	Pink, irregular, dendritic, flat, mucoid, opaque
BSL2	Light-yellow, irregular, dendritic, flat, mucoid, opaque
BSL3	Light-brown, irregular, dendritic, flat, mucoid, opaque
BSL6	Pink, circular, convex, entire, mucoid, opaque
BSL7	Cream, spreading, flat, dry, opaque
BSL8	Deep-yellow, irregular, lobate, mucoid, opaque
BPSW5	Cream, spreading, flat, mucoid, transparent
BPSWB1	White, circular, convex, entire, mucoid, opaque
BPSWB6	White, circular, concave, lobate, dry, presence of hydrolysis zone, opaque
BPSWB7	Cream, circular, lobate, mucoid, presence of hydrolysis zone, mucoid
BPSWB8	Cream, circular, concave, entire, dry, opaque
SWJ1	Cream, irregular, spreading, mucoid, transparent, opaque
SWJ2	Cream, circular, convex, small-sized, entire, mucoid, opaque
SWJ4	Yellow, circular, convex, entire, opaque
SWJ6	White, irregular, mucoid, opaque
ICK2	Cream, circular, concave, mucoid, opaque
ICK4	Light-pink, circular, convex, entire, shiny, mucoid
IKK1	Cream, circular, convex, entire, mucoid, shiny, mucoid, opaque
IKK3	Cream, undulate, flat, dry, opaque

Table 2: Colony characteristics of the thermophilic bacterial strains

Strain code	Colony morphology
HS1	White, irregular, lobate, flat, slimy, presence of hydrolysis zone, opaque
HS2	Off-white, irregular, dendritic, flat, slimy, presence of hydrolysis zone
HS3	Cream, circular, flat, entire, dry, opaque
HS4	Cream, circular, convex, small-sized, entire, mucoid

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Screening of the thermophilic bacterial strains for lipase production using submerged fermentation technique

An inoculum was prepared by transferring a loop full of the pure organisms into 25 mL sterile nutrient broth and incubated on shaker at 50 °C for 48 h. After the incubation period, the cells in the broth were counted using Neubauer Haemocytometer Chamber. Two mL (1.0×10^{-6} cells/mL) of bacterial suspension was then transferred from the seed culture to 50 mL production medium (nutrient broth supplemented with 2% tributyrin) contained in 250 mL Erlenmeyer flask. The bacterial culture was allowed to grow for 48 h at 50 °C under static condition as well as on incubator shaker maintained at 150 rpm (Khudair, 2016). About 3 mL of the culture broth was taken out after the incubation period and optical density was measured at 540 nm wavelength using spectrophotometer (UV-VIS Shimadzu, Japan). After measurement, the culture broth was centrifuged at 8,000 rpm for 20 min at 4 °C and the supernatant was used as crude lipase for determination of lipase activity. All experiments were conducted in triplicate.

Lipase activity assay

Lipase activity was measured according to the method of Gutarra *et al.* (2009) with slight modification. Lipase assay reaction was carried out by addition of 0.05 mL of the crude enzyme to a solution of 2.2 mL of 25 mM phosphate buffer (pH 7.0) and 0.25 mL of 2.5 mM pNPL. The hydrolysis reaction was carried out at 60 °C for 10 min, after which 0.25 mL of 0.1M Na_2CO_3 was added to terminate the reaction and the activity was determined at 412 nm wavelength. One unit of lipase activity is defined as the amount of enzyme releasing 1 mol of p-nitrophenol per minute under standard assay conditions.

Molecular identification and phylogenetic analysis of the selected bacterial strains

Each of the thermophilic and halophilic selected bacterial strains with highest lipase producing potential upon submerged fermentation was outsourced for molecular identification by determination of 16S rRNA partial gene sequence. The 16S rRNA gene sequencing service for the strains was provided by MyTACG Bioscience Enterprise, Selangor, Malaysia. PCR amplification and partial sequencing of the 16S rRNA gene was performed. The gene sequence of the two selected strains was performed by direct sequencing of enzymatically amplified DNA template using specific universal primers sets of BSR1541 (5ˈAAG GAG GTG ATC CAG CCG CA 3ˈ) and BSF8 (5ˈ AGA GTT TGA TCC TGG CTC AG 3ˈ).

Phylogenetic analysis

The obtained 16S rRNA sequences of both halophilic and thermophilic strain were added to the available bacterial 16S rRNA sequences database; and the sequences were integrated into the database with the automatic alignment tool. The 16S rRNA partial sequence of the strains was analysed and compared to that of other bacteria in GenBank of National Centre for Biotechnology Information (NCBI). Phylogenetic and molecular evolutionary analyses for 16S rRNA gene nucleotide sequences were conducted for sequence alignments using the computer programs ClustalW and MEGA5 software. The phylogenetic trees for the halophilic and thermophilic strains were constructed by means of NCBI BLAST tree neighbour-joining method using NCBI BLASTN 2.2.31+ software package (Zhang *et al*., 2000). The statistical support for the tree branches was achieved through Bootstrap analysis.

RESULTS AND DISCUSSION

Isolation of halophilic and thermophilic bacteria

Hypersaline environments represent typical extreme habitats with high salt concentration, alkalinity, and low oxygen stress which may limit their biodiversity. Yet halophiles are found in many of such saline environments such as hypersaline waters and soils, salt deposits and in variety of salted products, ranging from salted animal hides, salted fish, or meats to fermented foods (Ventosa, 2006). Screening of bacteria from saline soil and water samples, salty effluents, and salted fish samples led to the isolation of 7 extremely halophilic bacteria and 38 moderately halophilic bacteria (a total of 45 halophilic strains). Amongst these strains, there were 25 Gram-positive rods, 10 Gram-negative rods, 8 Gram-positive cocci and 2 Gram-negative cocci. Non-halophilic bacteria were not among the strains isolated probably due to the hypersaline condition of the samples which is not suitable for their inhabitance.

Thermophiles on the other hand are organisms growing at elevated temperatures typically associated with solar, geothermally, industrially or biological heated environment and may perform novel metabolic functions producing highly stable enzymes and proteins capable of significant functions adaptable to industrial and biotechnological applications (Norashirene *et al.*, 2012). A total of 4 thermophilic bacterial strains were isolated from Felda residence hot spring (Perak) and selected based on microscopy and colony morphology (Table 1) on nutrient agar. Norashirene *et al.* (2012) and Adhikari *et al.* (2015) also reported isolation of some thermophilic bacteria from hot springs of Hulu Langat, Selangor, Malaysia and Tatopani, Bhurung, Nepal respectively. The pure cultures of the strains were maintained on respective agar slants for further investigation.

Morphological and physiological characterization of halophilic and thermophilic bacteria

Morphological appearance of the halophilic bacteria on modified SGC isolation agar plate showed different colony colours ranging from cream, yellow, brown, pink to white

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(as seen in Table 1). Colonies of different shape, sizes, colour, texture, elevation, and margin were formed by the isolates. Colony characterization of these halophiles indicates that they are highly diversified. The isolated halophilic strains were predominantly Gram-positive (33) and only 12 were Gram-negative.

Physiological characteristics of the halophilic bacterial strains are presented in Table 3. Micro-morphological

Table 3: Physiological characteristics of the halophilic bacterial strains.

Strain code	Morph-ology	Gram reaction	NaCl Concentration						Temperature					
			0M	1M	2M	3M	4M	5M	35°C	40°C	45°C	50°C	55°C	60°C
BSO1	Cocci	+	+	+	+	-	-	-	+	+	+	+	-	-
BSO2a	Rods	+	+	+	+	-	-	-	+	+	+	-	-	-
BSL1	Rods	+	+	+	+	+	-	-	+	+	+	+	-	-
BSL2	Rods	+	+	+	+	-	-	-	+	+	+	-	-	-
BSL3	Short rods	+	+	+	+	-	-	-	+	+	+	-	-	-
BSL6	Long rods	+	+	+	+	+	-	-	+	+	+	+	-	-
BSL7	Short rods	+	+	+	+	-	-	-	+	+	+	+	+	+
BSL8	Rods	+	+	+	+	-	-	-	+	+	+	+	+	-
BPSW5	Short rods	-	+	+	+	+	+	-	+	+	+	-	-	-
BPSWB1	Cocci	+	+	+	+	+	+	-	+	+	+	-	-	-
BPSWB6	Short rods	+	+	+	+	-	-	-	+	+	+	+	+	-
BPSWB7	Cocci	+	-	+	-	-	-	-	+	+	+	+	+	-
BPSWB8	Long rods	-	+	+	+	-	-	-	+	+	+	+	-	-
SWJ1	Long rods	+	+	+	+	-	-	-	+	+	+	+	+	-
SWJ2	Short rods	-	-	+	+	-	-	-	+	+	+	-	-	-
SWJ4	Small rods	-	+	+	-	-	-	-	+	+	+	-	-	-
SWJ6	Small rods	-	+	+	+	-	-	-	+	+	+	-	-	-
ICK2	Cocci	+	+	+	+	+	-	-	+	+	+	+	+	-
ICK4	Cocci	-	+	+	+	-	-	-	+	+	-	-	-	-
IKK1	Long rods	+	+	+	+	-	-	-	+	+	+	+	+	+
IKK3	Rods	+	+	+	+	-	-	-	+	+	+	+	+	+

(+) Positive reaction; (-) Negative reaction

Table 4: Micro-morphological and physiological characteristics of thermophilic bacterial strains from hot spring.

Strain code	Morphology	Gram reaction
HS1	Small rods	+
HS2	Small rods	+
HS3	Long rods	+
HS4	Small rods	+

characterization and Gram's reaction of the strains was done with reference to Bergey's Manual of Determinative Bacteriology. Observation under the microscope showed cells with different morphologies; long and short rods, coccibacilli and cocci types. The halophilic strains were tested for salinity tolerance. All the strains showed growth in sodium chloride (NaCl) medium. Most of these strains performed best at 0-3M NaCl concentration indicating they are moderate halophiles. Ghasemi *et al.* (2011) and Babavalian *et al.* (2013) earlier isolated moderate halophilic lipase producing bacteria from various salty environments. However, 7 of the isolated strains showed no growth in the absence of NaCl. Thus indicating they are strict halophiles (Khunt, 2012). The temperature tolerance of the strains was tested at 35 °C, 40 °C, 45 °C, 50 °C, 55 °C, and 60 °C. All the strains performed well at 35 °C, 40 °C, and 45 °C, with few showing good growth at 50 °C and 55 °C, and only four (BSL7, IMT2, IKK1, and IKK3) showed growth at 60 °C.

In this study, morphological and physiological characterization of the thermophilic bacterial strains were also performed. From the colony characteristics of the thermophilic bacteria presented in Table 2, it is shown that the colonies of strain HS1 and HS2 are irregular in shape, white and off-white in colour respectively. Conversely, colonies of strain HS3 and HS4 are circular with entire edge and cream in colour. Further investigation on micro-morphological and physiological properties (result presented in Table 4) of these strains (HS1, HS2, HS3, and HS4) showed that they are all Gram positive non-motile rods. For more characterization and identification of thermophilic and halophilic strains, a combination of phenotypic and genotypic methods is required for identification of the strains (Khalil, 2011), which will be conducted in the next phase of this research.

Screening of thehalophilic and thermophilic bacterial strains for lipase production

Both the halophilic and thermophilic bacterial strains were first screened primarily on triglyceride (tributyrin) containing solid agar for potential lipolytic ability and were further screened for lipase production in submerged fermentation broth.

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Screening of lipase producing bacterial strains on solid agar

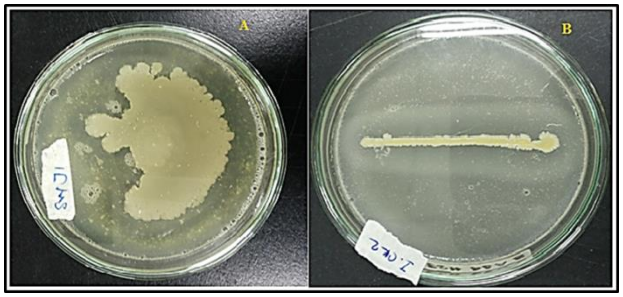

Figure 1: Primary screening of halophilic lipase producing bacterial strains (A) SWJ1 and (B) ICK2 on tributyrin agar plate.

Screening of microorganisms for lipolytic activity is relatively easy and is mostly performed on agar plates containing triglycerides (Sebdani et al., 2011). Screening of the lipase producing halophilic bacteria was done on modified SGC agar plates containing 1% tributyrin as substrate. Agar plate screening of moderate halophilic bacteria for lipase activity using tributyrin (1%) was earlier reported in the study of Ghasemi *et al.* (2011). The result of screening halophilic bacterial strains for lipase production is presented in Table 5. Lipase producing organisms were detected on the basis of zone (substrate utilization) surrounding the colonies. Among the 45 strains screened for lipase activity, 21 strains were positive for lipase activity on tributyrin agar plate.

No lipolytic activity was detected in 24 strains screened on the tributyrin-containing agar. Strain SWJ1 had the highest value (7.00) for relative enzyme activity (REA) in tributyrin-containing agar plate follow in order by strain ICK2 with relative enzyme activity of 6.88. Thus indicating that these strains exhibited the highest lipase producing potential. Figure 1 shows halophilic strains SWJ1 and ICK2 with the high lipase producing potential on tributyrin agar plate respectively. The figure showed that strains SWJ1 and ICK2 display a clear halo zone around the colony on tributyrin agar after 72 h. Clear zones around the strains indicate hydrolysis activity of lipolytic enzyme on tributyrin substrate. Tributyrin consists of short chain fatty acid, butyric acid which is generally used for screening of lipase and estimation of lipase activity (Nerurkar *et al.*, 2013). Based on the clear halo zone and opaque white zones around the colonies, 21 halophilic strains with considerable lipase producing potential were selected for lipase production evaluation in secondary screening.

Thermophilic bacterial strains were also screened for extracellular lipase on solid tributyrin agar medium. Table 6 shows that all the strains had considerable lipase activity but strain HS4 had the highest (1.80) REA value, thus indicating higher lipase activity at this screening stage. Lipases breakdown tributyrin, a triglyceride which results in the formation of clear zone surrounding the bacterial growth (Ghazali and Hamid, 2015). Based on the lipolytic potential of these strains on primary screening agar medium, they were further screened on liquid fermentation broth.

Table 5: Screening halophilic lipase producing-bacterial strains on modified Sehgal and Gibbons complex-Tributyrin agar.

	Tributyrin
Strain code	**Ratio of opaque zone diameter (mm) and colony diameter (mm)**
BSO1	1.56
BSO2A	5
BSO2B	-
BSL1	1.42
BSL2	5
BSL3	3.12
BSL4	-
BSL5	-
BSL6	2
BSL7	1.21
BSL8	1.4
BPSW1	-
BPSW2	-
BPSW3	-
BPSW4	-
BPSW5	1.09
BPSW6	-
BPSW7	-
BPSW8	-
BPSWB1	1.5
BPSWB2	-
BPSWB3	-
BPSWB4	-
BPSWB5	-
BPSWB6	1.13
BPSWB7	1.43
BPSWB8	1.5
BPWSB9	-
SWJ1	7
SWJ2	2.67
SWJ3	-
SWJ4	1.29
SWJ6	1.8
IGK1	-
IGK2	-
IGK3	-
IMT1	-
IMT2	-
ICK1	-
ICK2	6.88
ICK3	-
ICK4	1.4
IKK1	1.22
IKK2	-
IKK3	1.08

Secondary screening for lipase production in liquid fermentation broth

Twenty-one halophilic strains with high extracellular lipase producing potential were selected for the determination of lipase activity in liquid fermentation broth under static and agitation condition. Upon cultivation, a moderate

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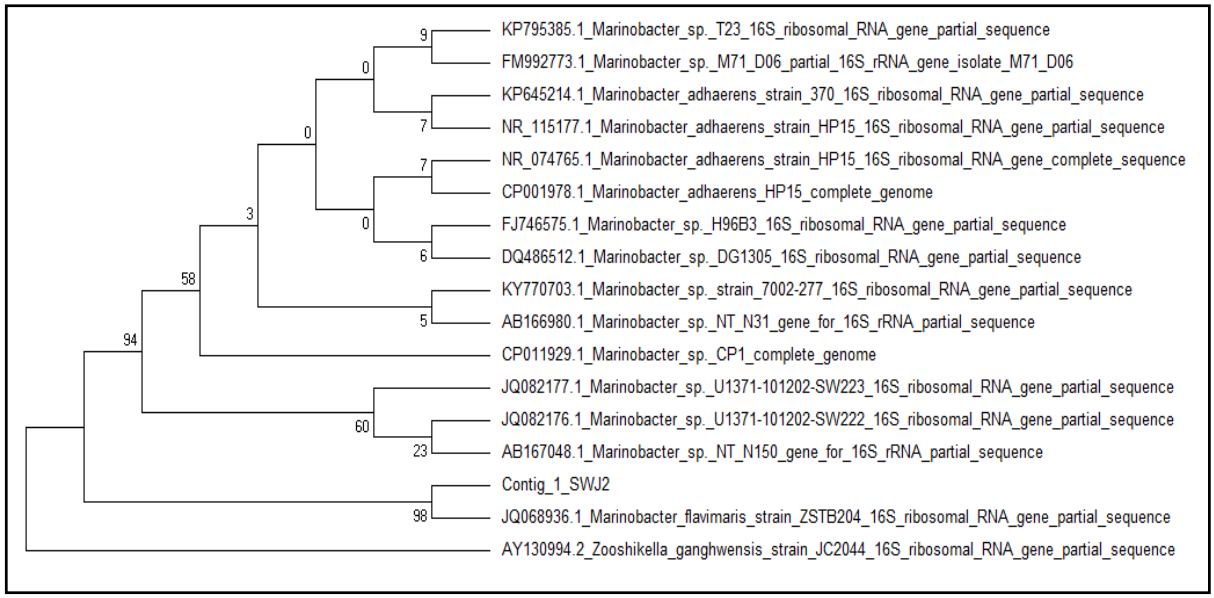

Figure 2: Phylogenetic relationships of a moderate halothermophilic lipase producing *Marinobacter* sp. SWJ2 isolated from Seawater in Tanjung Piai National park Johor Bahru, Malaysia. The phylogenetic tree based on 16S rRNA partial gene sequence was constructed by the neighbour-joining method. Accession numbers of the sequences used in the study precede the strain designations as shown in the diagram. *Zooshikella ganghwenssi* JC2044 (AY130994.2) was used as an outgroup in the analysis.

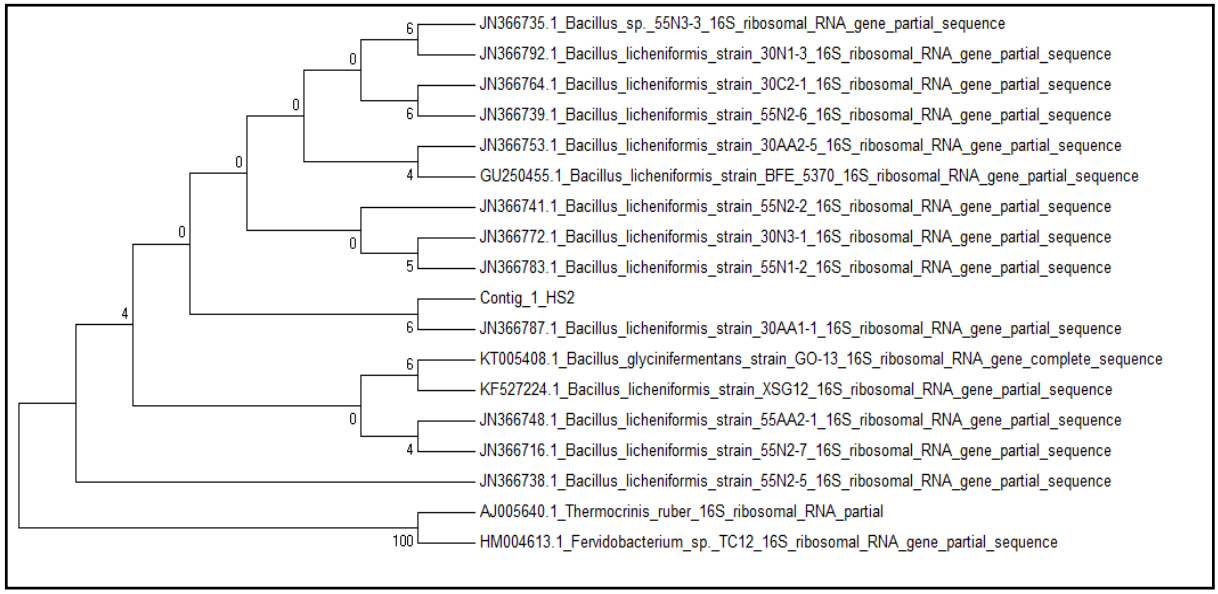

Figure 3: Phylogenetic relationships of a thermophilic lipase producing *Bacillus* sp. HS2 isolated from FELDA residence hot spring Sungkai, Perak, Malaysia. The phylogenetic tree based on 16S rRNA partial gene sequence was constructed by the neighbour-joining method. Accession numbers of the sequences used in the study precede the strain designations as shown in the diagram.

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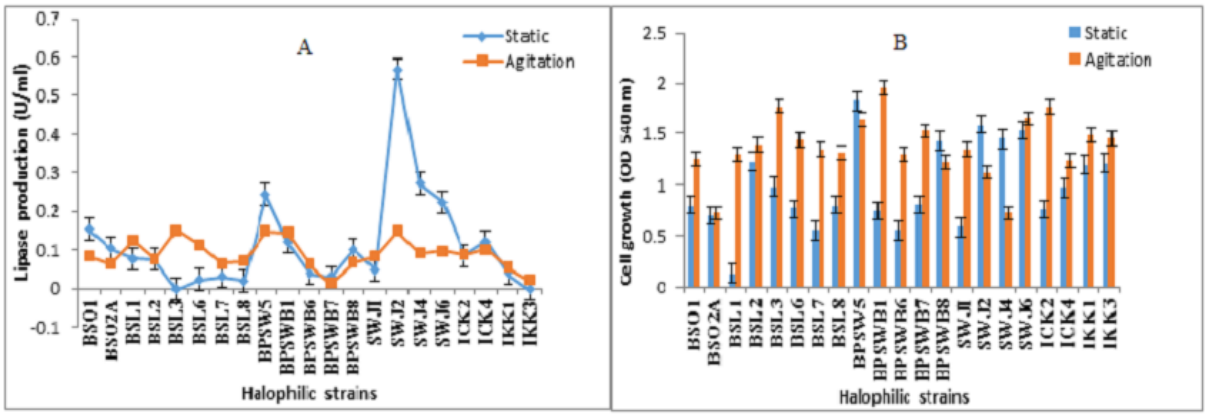

Figure 4: A. Lipase production and B. cell growth of halophilic bacteria in secondary screening fermentation broth.

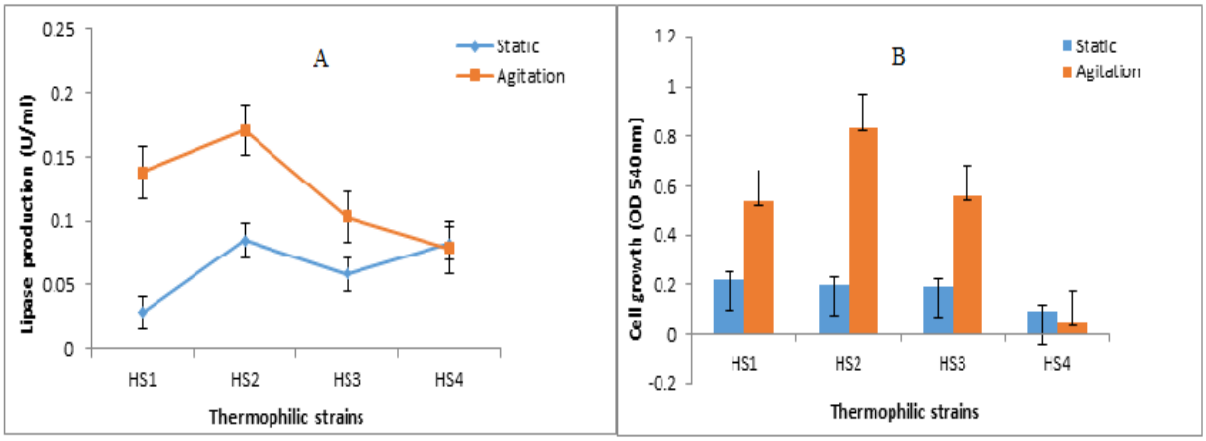

Figure 5: A. Lipase production and B. cell growth of thermophilic bacteria in secondary screening fermentation broth.

halophilic strain SWJ2 gave the best lipase production (0.57 U/mL) under static condition of fermentation followed in order by SWJ4 and BPSW5 with 0.27 U/mL and 0.24 U/mL lipase production respectively under the same condition as seen in Figure 4. Strain BSL3 had the highest lipase production (0.15 U/mL) under agitation condition but still fall short of performance when compared with SWJ2 strain which obviously had a better lipase production (0.57 U/mL) under static fermentation condition. Earlier study by Namwong *et al.* (2015) also reported a moderate halophilic strain C15-2 as highest lipase producer (2.5 U/mL) during secondary screening of halophilic bacterial strains in liquid culture medium. Studies about determination of halophilic extracellular enzymes have reported that most moderate halophilic bacteria have very good hydrolytic capacity (Rodríguez-Herrera *et al.*, 2014). Bacterial lipases are mostly extracellular, where the biggest factor in the expression of their activity has always been the requirement for an adequate carbon source and hydrolysable esters in the form of inducer, mainly triacylglycerols (de Guzmán, 2015). Figure 4 also shows cell growth of halophilic strains in liquid culture during screening of the strains for lipolytic activity. It could be observed that agitation contributed to bacteria growth rather than lipase production in most of the strains except for strains BPSW5, BPSWB8, SWJ2 and SWJ4. Coincidentally, strains BPSW5, BPSWB8, SWJ2 and SWJ4 showed better lipase production under static condition. In this study, lipase production was favoured by static condition while cell biomass was favoured by agitation. The increase in biomass under agitation condition might be due to beneficial influence on cell morphology, that is, cell growth, as a result of dissolved oxygen availability in the fermentation broth. However, the variation in lipase production might be due to different rate of substrate utilization at different fermentation condition which ultimately influenced the product yield (Thakur *et al.*, 2014).

All the thermophilic strains screened on agar plate culture for lipolytic potential determination were equally screened for their ability to produce lipase in liquid culture with 1% tributyrin for a period of 48 h. The result in Figure 5 shows that low lipase production ranging from 0.003-

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0.085 U/mL was recorded for all the strains under static condition. However, substantial amount of lipase was observed for all the strains under agitation condition where HS2 strain had the highest (0.171 U/mL) lipase production. Stathopoulou *et al.* (2013) previously recorded similar amount of lipase during screening of some thermophilic bacteria in liquid culture for lipase production. The cell growth of the thermophilic strains (as seen in Figure 5) was equally enhanced by agitation condition where strain HS2 recorded highest biomass (0.84) and the other strains HS3, HS1 and HS4 had 0.56, 0.53 and 0.05 respectively. Agitation and aeration condition in a fermenter play important role in growth of bacteria and production of thermostable lipase enzyme. Agitation is an important parameter for ensuring nutrient availability (especially carbon sources) in a growth medium and also promotes effective mass transfer to the liquid medium in the fermenter (Balan *et al.*, 2010). Generally in this study, static fermentation condition favoured lipase production by halophilic strains while agitation improved lipase production by thermophilic bacteria strains.

Table 6: Screening the thermophilic lipase producing-bacterial strains on Tributyrin-nutrient agar.

Strain code	Ratio of opaque zone diameter (mm) and colony diameter (mm)
	Tributyrin
HS1	1.19
HS2	1
HS3	1.67
HS4	1.8

Molecular identification of the halophilic and thermophilic bacteria

Out of all the 45 halophilic strains, only one (SWJ2) with high lipase producing potential in the fermentation broth was selected for identification based on 16S rRNA gene sequencing. On the other hand, amongst thermophilic strains, HS2 with better lipase production performance was equally selected for 16S rRNA gene sequencing.

Phylogenetic analysis based on 16S rRNA partial gene sequence comparisons (as seen in Figure 2) showed that the strain SWJ2 belonged to the genus *Marinobacter* and showed 99% similarity with *Marinobacter flavimaris* ZSTB204. Thus, the strain was tentatively named as *Marinobacter* sp. SWJ2 and its gene sequence has been deposited in the GenBank; and assigned accession number JQ068936.1. *Marinobacter* sp. EMB 5,6,7,8 (Kumar *et al.*, 2012), *M. lipolyticus* (Martin *et al.*, 2003) have previously been isolated and reported as moderately halophilic potent lipase producers.

Furthermore, the Basic Local Alignment Search Tool (BLAST) result of the partial 16S rRNA gene sequence of the candidate strain HS2 (Figure 3) showed that the strain belong to the genus *Bacillus* and showed 99% homology with *B. licheniformis* 30AA1-1. Therefore, strain HS2 was tentatively named as *Bacillus* sp.HS2 and its 16S rRNA sequence has been deposited in the GenBank with accession number JN366787.1. Previous studies also reported the presence and isolation of thermophilic Bacilli species belonging to the genera *Bacillus*, *Geobacillus* and *Paenibacillus* from different geothermal springs as thermostable lipase producers (Ghati *et al.*, 2013; Lele and Deshmukh, 2016).

CONCLUSION

An investigation on the lipolytic potential of some halophilic and thermophilic bacterial strains was performed. It was observed during screening in liquid culture that a moderate halo-thermophile, *Marinobacter* sp. SWJ2 had maximum lipase production of 0.569 U/mL under static condition of fermentation while a thermophile, *Bacillus* sp. HS2 had maximum lipase production of 0.171 U/mL under agitation condition. The screening results obtained in the present study suggest that these two strains (*Marinobacter* sp.SWJ2 and *Bacillus* sp. HS2) with varying physiological properties possess high lipolytic potential and represents promising candidates for high temperature lipolytic industrial applications.

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REFERENCES

Adhikari, H., Ghimire, S., Khatri, B. and Yuvraj, K. C. (2015). Enzymatic screening and molecular characterization of thermophilic bacterial strains isolated from hot spring of Tatopani, Bhurung, Nepal. *International Journal of Applied Sciences and Biotechnology* **3(3), 392-397.**

Anisha, C., Mathew, J. and Radhakrishnan, E. K. (2012). Extracellular lipolytic enzyme production by a novel extremely halophilic bacterium. *Universal Journal of Environmental Research and Technology* **2(3), 143-148.**

Arora, S., Vanza, M. J., Mehta, R., Bhuva, C. and Patel, P. N. (2014). Halophilic microbes for bio-remediation of salt affected soils. *African Journal of Microbiology Research***8, 3070-3078.**

Babavalian, H., Amoozegar, M. A., Pourbabaee, A. A., Mohgaddam, M. M. and Shakeri, F. (2013). Isolation and identification of moderately halophilic bacteria producing hydrolytic enzymes from the largest hypersaline playa in Iran. *Microbiology* **82(4), 466-474.**

Balan, A., Magalinggam, N., Ibrahim, D. and Rahim, R. (2010). Thermostable lipase production by *Geobacillus thermodenitrificans* in a 5-L stirred-tank bioreactor. *Internet Journal of Microbiology* **8(2), 1-9.**

Bisht, S. S. and Panda, A. K. (2011). Biochemical characterization and 16SrRNA sequencing of few lipase-producing thermophilic bacteria from Tatopani hot water spring, Orissa, India. *Biotechnology Research International* **11, 452710-452715.**

Calimlioglu, B. and Arga, K. Y. (2014). Proteins from halophilic bacteria: purification and their applications.*In*: Protein Purification - Principles and Trends, Edition 1. I Concept Press Ltd, Brisbane, Australia. **pp. 145-168.**

DasSarma, S. and DasSarma, P. (2012). Halophiles. *In*: eLS. University of Maryland, Baltimore, Maryland, USA. John Wiley and Sons, Ltd: Chichester. **pp. 85-100.**

de Guzmán, M. N. (2015). Lipolytic enzyme production by halophilic/halotolerant microorganisms isolated from Laguna Verde, Bolivia. *Revista Boliviana de Química* **25(1), 14-23.**

Delgado-García, M., Valdivia-Urdiales, B., Aguilar-González, C. N., Contreras-Esquivel, J. C. and Rodríguez-Herrera, R. (2012). Halophilic hydrolases as a new tool for the biotechnological industries. *Journal of the Science of Food and Agriculture* **92(13), 2575-2580.**

Dheeman, D. S., Frias, J. M. and Henehan, G. T. (2010). Influence of cultivation conditions on the production of a thermostable extracellular lipase from *Amycolatopsis mediterranei* DSM 43304. *Journal of Industrial Microbiology and Biotechnology* **37(1), 1-17.**

Fuciños, P., González, R., Atanes, E., Sestelo, A. B. F., Pérez-Guerra, N., Pastrana, L. and Rúa, M. L. (2012). Lipases and esterases from extremophiles: Overview and case example of the production and purification of an esterase from *Thermus thermophilus* HB27. *Lipases and Phospholipases: Methods and Protocols* **861,239-266.**

Ghasemi, Y., Rasoul-Amin, S., Kazemi, A., Zarrini, G., Morowvat, M. H. and Kargar, M. (2011). Isolation and characterization of some moderately halophilic bacteria with lipase activity. *Microbiology* **80(4), 483-487.**

Ghati, A., Sarkar, K. and Paul, G. (2013). Isolation, characterization and molecular identification of estereolytic thermophilic bacteria from an Indian hot spring. *Current Research in Microbiology and Biotechnology* **1(4), 196-102.**

Ghazali, S. A. M. and Hamid, T. H. T. A. (2015). New lipase producing β-proteobacteria strains *Caldimonas* sp. and *Tepidimonas* sp. Isolated from a Malaysian Hot Springs. *Sains Malaysiana* **44(5), 701-706.**

Gutarra, M. L. E., Godoy, M. G., Maugeri, F., Rodrigues, M. I., Freire, D. M. G. and Castilho, L. R. (2009). Production of acidic and thermophilic lipase of the mesophilic fungus *Penicillium simplicissimum* by solid state fermentation. *Bioresource Technology* **100(21), 5249-5254.**

Kanlayakrit, W. and Boonpan, A. (2007). Screening of halophilic lipase-producing bacteria and characterization of enzyme for fish sauce quality improvement. *Kasetsart Journal (Natural Sciences)* **41, 576-585.**

Karan, R., Capes, M. D. and DasSarma, S. (2012). Function and biotechnology of extremophilic enzymes in low water activity. *Aquatic Biosystems* **8(1), 1-4.**

Khalil, A. (2011). Screening and characterization of thermophilic bacteria (lipase, cellulase and amylase producers) from hot springs in Saudi Arabia. *Journal of Food, Agriculture and Environment* **9(2), 672-675.**

Khudair, S. H. (2016). Isolation and optimization of thermophilic lipase producing bacteria from soil contaminated with used engines oil. *Wasit Journal for Science and Medicine* **8(4), 66-74.**

Khunt, M. D. 2012. Biodiversity study of halophiles from little Rann of Kutch with special emphasis on lipase production. A PhD thesis. Faculty of Science (Microbiology), Saurashtra University, Rajkot.

Kumar, S., Karan, R., Kapoor, S., Singh, S. and Khare, S. (2012). Screening and isolation of halophilic bacteria producing industrially important enzymes. *Brazilian Journal of Microbiology* **43(4), 1595-1603**.

Lele, O. H. and Deshmukh, P. V. (2016). Isolation and characterization of thermophilic *Bacillus* sp. with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India. *Indian Journal of Applied Research* **2(5), 427-430.**

López-López, O., Cerdán, M. E. and González-Siso, M. I. (2015).*Thermus thermophilus* as a source of thermostable lipolytic enzymes. *Microorganisms* **3(4), 792-808.**

Martin, S., Márquez, M., Sánchez-Porro, C., Mellado, E., Arahal, D. and Ventosa, A. (2003).*Marinobacter lipolyticus* sp. nov., a novel moderate halophile with lipolytic activity. *International Journal of Systematic and Evolutionary Microbiology* **53(5), 1383-1387.**

Munawar, N. and Engel, C. P. (2013). Halophilic enzymes: Characteristics, structural adaptation and potential applications for biocatalysis. *Current Biotechnology* **2(4), 334-344.**

Namwong, S., Pensuk, W. and Pentasen, K. (2015). Studies of lipase production from halophilic bacteria. *Academic Journal of Science* **4(1), 265-270.**

Nerurkar, M., Joshi, M. and Adivarekar, R. (2013). Use of sesame oil cake for lipase production from a newly marine isolated *Bacillus sonorensis*. *Innovative Romanian Food Biotechnology* **13, 11-17.**

Norashirene, M., Rosliza, S. A., Sharina, M. N. and Fithriah, M. N. (2012). Screening and isolation of lipolytic, amylolytic and cellulolytic thermophiles from Hulu Langat hot spring, Selangor. *In*: Business Engineering and Industrial Applications Colloquium (BEIAC), 2012 IEEE.**pp.204-209.**

Olutiola, P. O., Famurewa, O. and Soutang, H. G. (2000). An Introduction to General Microbiology (A practical approach). Reprinted in Nigeria by Bolabay Press Ltd. **pp. 157-175.**

Patil, K. J., Chopda, M. Z. and Mahajan, R. T. (2011). Lipase biodiversity. *Indian Journal of Science and Technology* **4(8), 971-982.**

Rodríguez-Herrera, R., Delgado-García, M., Aguilar, C. and Contreras-Esquivel, J. (2014). Screening for extracellular hydrolytic enzymes production by different halophilic bacteria. *Mycopath* **12(1), 17-23.**

Salihu, A. and Alam, M. Z. (2014). Thermostable Lipases: an Overview of production, purification and characterization. *Biosciences Biotechnology Research Asia* **11(3), 1095-1107.**

Salihu, A. and Alam, M. Z. (2015). Solvent tolerant lipases: A review. *Process Biochemistry* **50(1), 86-96.**

Sebdani, R., Ardakani, M., Ghezelbash, G. and Sadrinasab, M. (2011). Phylogenetic characterization of lipase producing *Bacillus* strains isolated from Persian Gulf sediments. *Australian Journal of Basic and Applied Sciences* **5(4), 121-126.**

Sharma, R., Thakur, V., Sharma, M. and Birkeland, N. K. (2013). Biocatalysis through thermostable lipases: Adding flavor to chemistry. *Thermophilic Microbes in Environmental and Industrial Biotechnology*, Springer Netherlands. **pp.905-927**.

Stathopoulou, P. M., Savvides, L. A., Karagouni, A. D. and Hatzinikolaou, D. G. (2013). Unravelling the lipolytic activity of thermophilic bacteria isolated from a volcanic environment. *BioMed Research International* **12, 703130-703143.**

Thakur, V., Tewari, R. and Sharma, R. (2014). Evaluation of production parameters for maximum lipase production by *P. stutzeri* MTCC 5618 and scale-up in bioreactor. *Chinese Journal of Biology***13, 208462-208476.**

Ventosa, A. (2006). Unusual micro-organisms from unusual habitats: hypersaline environments. *In*: *Symposia-Society for General Microbiology*: Cambridge; Cambridge University Press; 1999**. Vol.66, pp.223.**

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **13(1), 203-214.**

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